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Factor XII Regulates the Pathological Process of Thrombus Formation on Ruptured Plaques

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Factor XII Regulates the Pathological Process of Thrombus Formation on Ruptured Plaques

Marijke J.E. Kuijpers, Paola E.J. van der Meijden, Marion A.H. Feijge, Nadine J.A. Mattheij, Frauke May, José Govers-Riemslag, Joost C.M. Meijers, Johan W.M. Heemskerk, Thomas Renné, Judith M.E.M. Cosemans

Objective—Atherothrombosis is the main cause of myocardial infarction and ischemic stroke. Although the extrinsic (tissue factor–factor VIIa [FVIIa]) pathway is considered as a major trigger of coagulation in atherothrombosis, the role of the intrinsic coagulation pathway via coagulation FXII herein is unknown. Here, we studied the roles of the extrinsic and intrinsic coagulation pathways in thrombus formation on atherosclerotic plaques both in vivo and ex vivo.

Approach and Results—Plaque rupture after ultrasound treatment evoked immediate formation of subocclusive thrombi in the carotid arteries of *Apoe*$^{-/-}$ mice, which became unstable in the presence of structurally different FXIIa inhibitors. In contrast, inhibition of FVIIa reduced thrombus size at a more initial stage without affecting embolization. Genetic deficiency in FXII (human and mouse) or FXI (mouse) reduced ex vivo whole-blood thrombus and fibrin formation on immobilized plaque homogenates. Localization studies by confocal microscopy indicated that FXIIa bound to thrombi and fibrin particularly in luminal-exposed thrombus areas.

Conclusions—The FVIIa- and FXIIa-triggered coagulation pathways have distinct but complementary roles in atherothrombus formation. The tissue factor–FVIIa pathway contributes to initial thrombus buildup, whereas FXIIa bound to thrombi ensures thrombus stability. **(*Arterioscler Thromb Vasc Biol*. 2014;34:1674-1680.)**

Key Words: blood coagulation ■ blood platelets ■ factor XII ■ fibrin ■ plaque rupture ■ thrombus ■ tissue factor

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Atherothrombosis, characterized by atherosclerotic lesion disruption with superimposed thrombus formation, is the major cause of acute coronary syndromes and cardiovascular death. Predominantly, platelet-rich (white) thrombi are formed at the ruptured area, because platelet recruitment preferentially occurs at regions of high shear rate and disturbed flow, whereas maximal fibrin generation occurs in regions of low flow.[1] Activated platelets support the tissue factor (TF) pathway of blood coagulation by binding various (anti)coagulation factors.[2] There is emerging evidence that platelets also support the intrinsic coagulation pathway mediated by factors XII (FXII) and XI (FXI), for example, by releasing polyphosphates, although the exact mechanism is still unclear.[3–5] Better understanding of these multifaceted roles of platelets in coagulation stimulation can lead to new approaches to selectively inhibit the pathways most relevant in atherothrombosis.

See accompanying editorial on page 1607

Collagen type I is not only the most potent platelet activating component in atherosclerotic plaques by binding and activating the glycoprotein VI receptor,[6–8] but has also has been shown to bind to and activate FXII.[9] Several mouse studies using nonatherosclerotic vessels point to a consolidating role of the FXII pathway in arterial thrombus formation and thrombus stabilization, as concluded from experiments with genetic ablation or pharmacological inhibition of FXII.[4,10–13] Interestingly, deficiency in FXII in man or mouse is not accompanied by abnormal bleeding.[3,14] On the contrary, individuals with partly reduced FXII levels have an increased risk of cardiovascular disease (reviewed in Renné et al[15] and Woodruff et al[16]), which indicates that the clinical consequences of (partial) FXII deficiency are more complex than the reported antithrombotic effects of FXII ablation in mice.

To date, no experimental data exist on a role of the intrinsic coagulation pathway in pathological thrombus formation after atherosclerotic plaque rupture. A recent report studying plaque-induced thrombin generation in vitro suggested that the intrinsic FXII pathway may not play a key role in this process, such in contrast to a predominant role for plaque-derived TF triggering the extrinsic pathway.[8] In the present article, we therefore compared the roles of both the extrinsic and intrinsic coagulation pathways in thrombus formation on atherosclerotic plaques in vivo and ex vivo.

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From the Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands (M.J.E.K., P.E.J.v.d.M., M.A.H.F., N.J.A.M., J.G.-R., J.W.M.H., J.M.E.M.C.); CSL Behring GmbH, Marburg, Germany (F.M.); Department of Experimental Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands (J.C.M.M.); Department of Plasma Proteins, Sanquin, Amsterdam, The Netherlands (J.C.M.M.); Clinical Chemistry, Department of Molecular Medicine and Surgery, Karolinska Institutet and University Hospital, Stockholm, Sweden (T.R.); and Department of Clinical Chemistry, University Medical Center Hamburg-Eppendorf, Hamburg, Germany (T.R.).

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Correspondence to Judith M.E.M. Cosemans, PhD, Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands. E-mail judith.cosemans@maastrichtuniversity.nl

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Nonstandard Abbreviations and Acronyms	
CTI	corn trypsin inhibitor
FVIIai	active-site inactivated factor VIIa
FXI	factor XI
FXII	factor XII
PRP	platelet-rich plasma
TF	tissue factor

Materials and Methods

Materials and methods are available in the online-only Supplement.

Results

Distinct Roles of Murine FXII and FVII Pathways in Arterial Thrombus Formation on Ruptured Atherosclerotic Plaques

In mouse models, the role of FXII in thrombus formation has only been investigated on damage of healthy arteries. Here, we studied the contribution of FXII to the thrombotic process after acute rupture of an atherosclerotic plaque, using a recently established model of acute plaque rupture in the carotid bifurcation of *Apoe*$^{-/-}$ mice.[17] In control *Apoe*$^{-/-}$ mice, ultrasound treatment of the plaque shoulder provoked rapid thrombus formation at the site of rupture (Figure 1A; Movie I in the online-only Data Supplement). The formed thrombi remained subocclusive and reduced in size because of platelet contraction and limited embolization during the first 10 minutes after rupture, but then became stable for at least several hours, as observed before.[17] In mice injected with one of the FXIIa inhibitors, corn trypsin inhibitor (CTI; 4 mg/kg) or recombinant infestin-4 human albumin fusion protein (30 mg/kg), thrombus formation tended to be reduced, an effect that became significant 1 to 2 minutes after plaque rupture (Figure 1A–1C). In addition, either inhibitor caused a significant increase in shedding of discernable emboli from the initial thrombus (Figure 1D; Movie II in the online-only Data Supplement).

Interestingly, injection of active-site inactivated FVIIa (FVIIai; 1 mg/kg), known to block murine TF–FVIIa interactions,[18] reduced thrombus formation at a more initial stage,

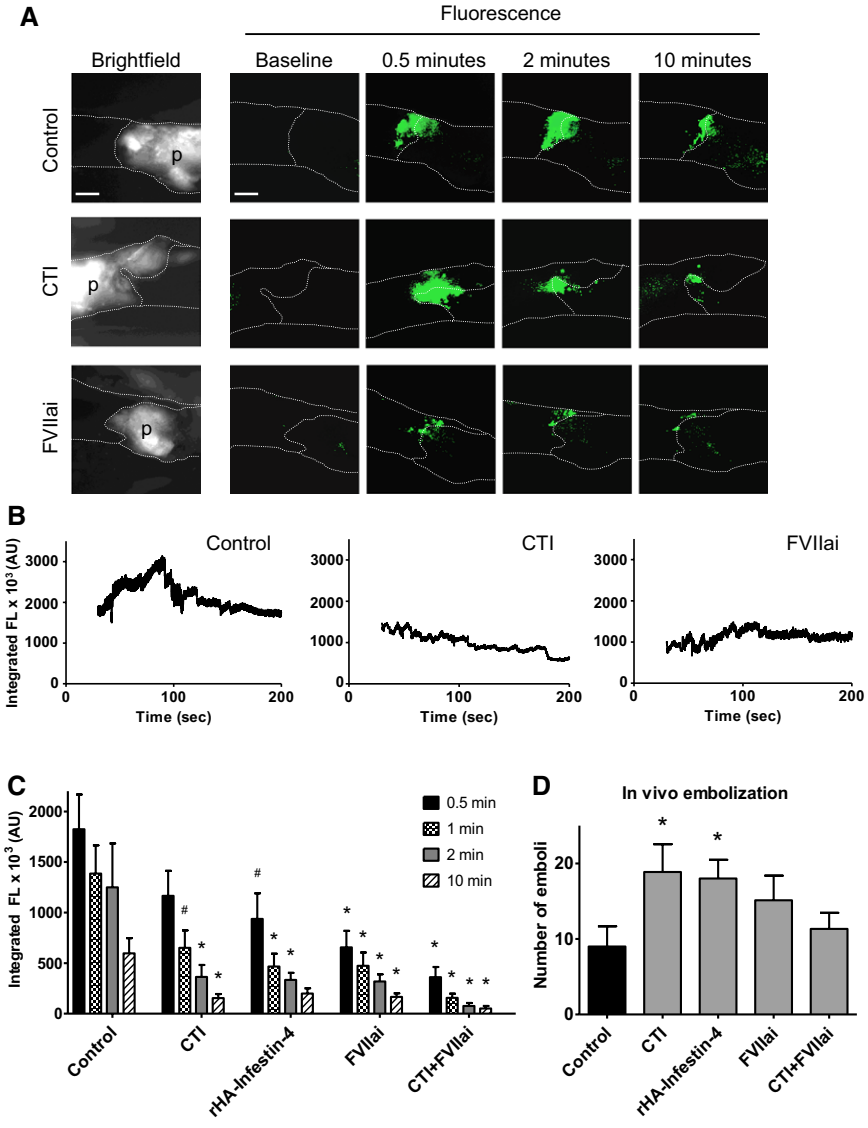

Figure 1. Distinct roles for factor XIIa (FXIIa) and FVIIa in arterial thrombus formation after acute plaque rupture in vivo. *Apoe*$^{-/-}$ mice were injected with saline (control), corn trypsin inhibitor (CTI; 4 mg/kg), recombinant infestin-4 human albumin fusion protein (rHA-infestin-4; 30 mg/kg), active-site inactivated FVIIa (FVIIai; 1 mg/kg), or CTI (4 mg/kg) and FVIIai (1 mg/kg). Carotid plaques were ruptured by ultrasound after which thrombus formation was quantified from preinjected CFSE-labeled platelets. **A**, Bright-field images of carotid plaque (p) and threshold masked fluorescence images of thrombi before and after ultrasound treatment. Dotted lines indicate contours of carotid artery and plaque (bars, 200 μm). **B**, Time courses of integrated carboxyfluorescein diacetate succinimidyl ester (CFSE) fluorescence (FL) intensity above background of thrombi formed (arbitrary units [AU]). Fluctuations in fluorescence intensity indicate embolus shedding. **C**, Quantification of thrombus size at various time points after ultrasound treatment (integrated fluorescence intensities from threshold masked images). **D**, Numbers of fluorescent emboli shed during 3 minutes after plaque rupture. Data are means±SE (n=7–11); **P*<0.05 and #*P*=0.051 compared with control.

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whereas embolization was not affected (Figure 1; Movie III in the online-only Data Supplement). In mice treated with FVIIai and CTI, thrombus formation at later, but not at early, time points was even further reduced (*P* values of 0.210, 0.074, 0.015, and 0.031 at *t*=0.5, 1, 2, and 10 minutes, respectively). Taken together, this pointed to distinct but complementary roles of the FVIIa- and FXIIa-triggered coagulation pathways in the thrombotic process after plaque rupture. Herein, TF–FVIIa interaction seems to be needed for initial thrombus formation, whereas FXII ensures thrombus stability.

Role of Murine FXII in Flow-Dependent Thrombus Formation on Atherosclerotic Plaque Material

To study the role of murine FXII in plaque-induced thrombus formation in more detail, flow experiments were performed where blood from wild-type or factor-deficient mice was perfused at moderately high shear rate under coagulant conditions (mmol/L Ca^{2+}/Mg^{2+}) over a surface coated with murine plaque material. With blood from wild-type mice, platelets rapidly adhered to the plaque and formed large thrombi. These thrombi contracted after several minutes, stained positively for exposed phosphatidylserine, and had fibrin fibers originating from them (Figure 2A). When using blood from *F12*$^{-/-}$ mice, initial platelet adhesion to the plaque material was unchanged, but ensuing thrombus formation and phosphatidylserine exposure (quantified as surface area coverage) were markedly diminished and no formed fibrin could visually be observed (Figure 2). Importantly, the defective thrombus formation was almost fully restored after supplementation with purified human FXII.

The main downstream effector protein of FXII is FXI which, however, can also be activated independently of FXII via a feedback loop of thrombin.[19,20] To study the role of FXI in plaque-induced thrombus formation, flow experiments were performed with blood from *F11*$^{-/-}$ and *F11*$^{-/-}$/*F12*$^{-/-}$ mice. In either case, the thrombus-forming process was similarly impaired as with *F12*$^{-/-}$ blood (Figure 2A, 2C, and 2D). Together, these data demonstrate a key role of murine FXII in thrombus and fibrin formation during whole-blood perfusion over a murine plaque surface mediated through the classical FXII–FXI reaction cascade.

Role of Human FXII in Flow-Dependent Thrombus Formation on Atherosclerotic Plaque Material

Having established the role of murine FXII in plaque-induced thrombus formation, we investigated the function of FXII also in the human system. Blood from control subjects and an FXII-deficient subject (FXII level <1%) was perfused over immobilized human plaque material under coagulation-promoting conditions. With the FXII-deficient blood, initial platelet adhesion to the plaque surface was comparable with that of control blood samples. However, after 10 minutes of perfusion, the surface area covered by thrombi, the amount of fibrin(ogen) binding, and the platelet procoagulant

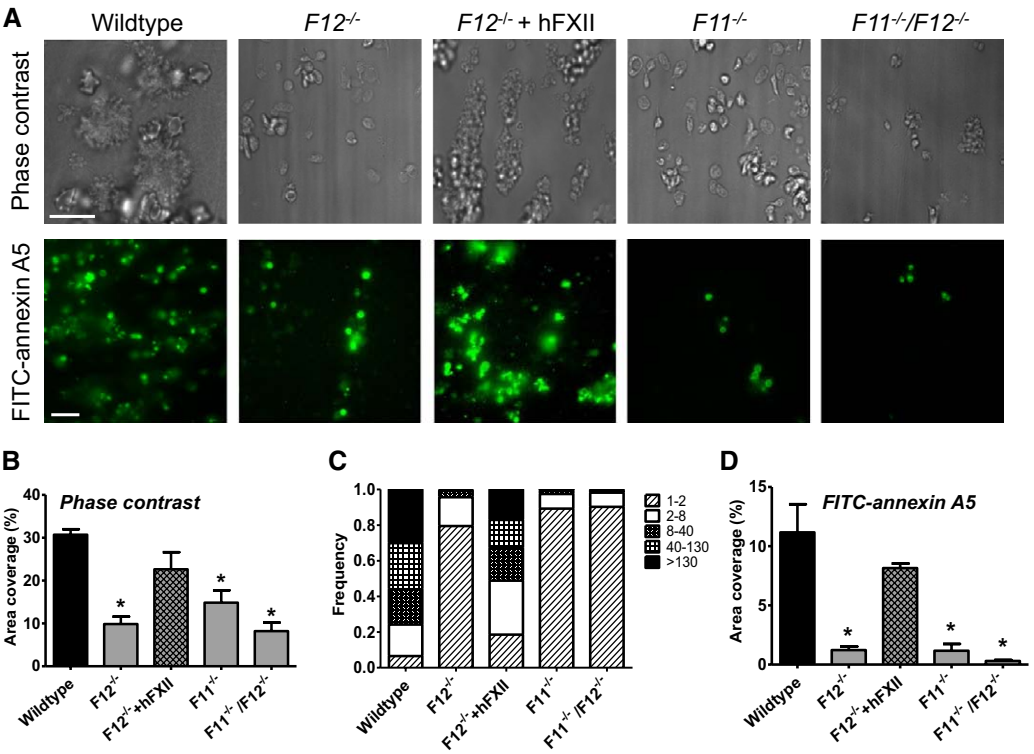

Figure 2. Deficiency of murine factor XI (FXI) and FXII impairs thrombus formation under coagulant conditions on plaque material ex vivo. Citrate-anticoagulated blood from *F11*$^{-/-}$, *F12*$^{-/-}$, *F11*$^{-/-}$*F12*$^{-/-}$ and corresponding wild-type mice was recalcified with $CaCl_2/MgCl_2$ and perfused over murine plaque material at a shear rate of 1000 s^{-1} for 6 minutes. Human FXII (hFXII; 375 nmol/L [30 μg/mL]) was added before the experiment when indicated. **A**, Representative phase-contrast images and fluorescence images of fluorescein isothiocyanate (FITC)–annexin A5–labeled procoagulant platelets (bars, 30 μm). **B**, Surface area coverage of deposited platelets. **C**, Frequency distribution of feature size of platelet–fibrin thrombi; estimated numbers of platelets per feature were 1 to 2, 2 to 8, 8 to 40, 40 to 130, and >130. **D**, Surface area coverage of phosphatidylserine-exposing platelets. Data are means±SE (n=4–5), **P*<0.05 vs corresponding wild type.

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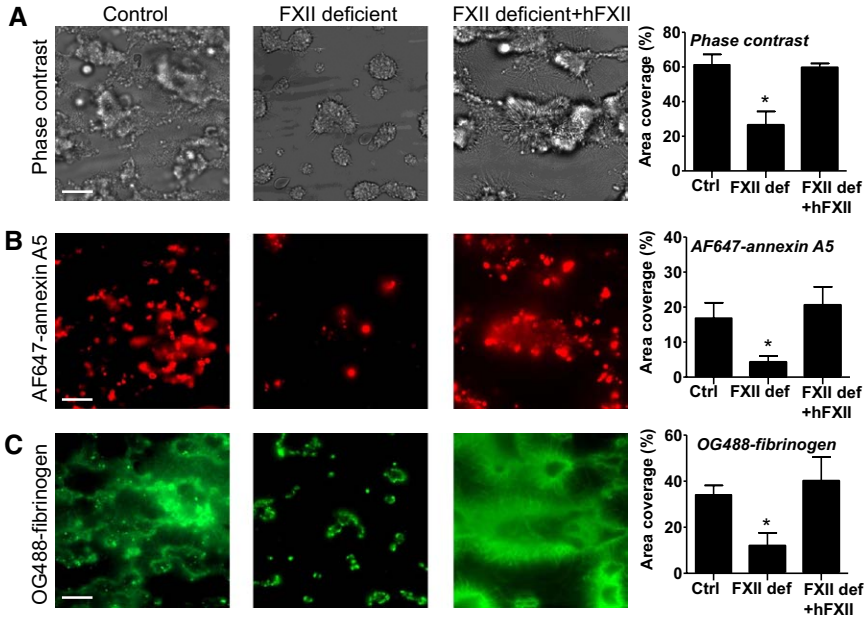

Figure 3. Deficiency of human factor XII (FXII) impairs thrombus formation under coagulant conditions on plaque material ex vivo. Citrate-anticoagulated blood from an FXII-deficient subject (FXII <1%) or from 3 healthy control (Ctrl) subjects was recalcified with $CaCl_2$/$MgCl_2$ and perfused over human plaque material at 500 s^{-1}. Where indicated, patient blood samples were supplemented with human FXII (hFXII; 375 nmol/L). Shown are representative microscopic images and quantitative data of surface area coverage analysis of (**A**) platelet–fibrin thrombi, (**B**) AF647–annexin A5 labeling of phosphatidylserine-exposing platelets, and (**C**) OG488-fibrin(ogen) labeling. Bars, 30 μm. Data are means±SE; **P*<0.05 vs Ctrl.

activity (phosphatidylserine exposure) were strongly reduced (Figure 3A–3C). Notably, addition of purified human FXII fully restored the defective thrombus formation and platelet procoagulant activity. Control measurements performed with Phe-Pro-Arg chloromethylketone-anticoagulated blood showed that thrombus formation on plaque material was highly similar with blood from control and FXII-deficient subjects at these thrombin-inhibiting/noncoagulating conditions (not shown).

To substantiate these findings, thrombus formation was assessed under coagulant conditions in control blood samples by pharmacological inhibition of FXIIa with CTI (50 μg/mL) or recombinant infestin-4 human albumin fusion protein (250 μg/mL). Either inhibitor caused a 50% reduction in surface area coverage and procoagulant activity of platelets (Figure 4A and 4B). Dose–response experiments with recombinant infestin-4 human albumin fusion protein (0.1–2.0 mg/mL) indicated an optimal inhibitory effect at 250 μg/mL (not shown). Incubation of the blood with FVIIai (10 μg/mL) resulted in a significant reduction in thrombus formation and in a trend toward reduced platelet procoagulant activity (Figure 4).

Role of Human FXII in Plaque-Mediated Thrombin Generation and Clotting in Platelet-Rich Plasma

To confirm the effects of FXII inhibition and blockage of TF–FVIIa interaction on platelet- and plaque-dependent coagulation, clotting times were measured in recalcified human platelet-rich plasma (PRP). Addition of plaque homogenate reduced the time to Ca^{2+}-triggered clot formation, which effect was partly antagonized with either CTI or FVIIai and fully antagonized by application of both inhibitors (Table I in the online-only Data Supplement). These data thus point to a key role of human FXII in fibrin clot formation in PRP, triggered by plaque material.

Knowing that thrombin provides a positive feedback loop by stimulation of platelet phosphatidylserine exposure and hence of platelet procoagulant activity,[2] we examined in more detail the contribution of FXII to thrombin generation in PRP triggered by $CaCl_2$ with(out) plaque material. The thrombin generation curve generated in control PRP in the presence of $CaCl_2$ was absent in FXII-deficient PRP (Table II in the online-only Data Supplement). In control PRP, plaque material caused a similar acceleration and increase in thrombin generation as collagen (Figure 5A). In contrast, in FXII-deficient PRP, plaque material and collagen triggered a slow-onset, low thrombin generation, whereas the addition of purified FXII normalized this process (Figure 5B). Thus, we concluded that FXII plays a key stimulating role in plaque-induced and platelet-dependent thrombin generation.

Localization of FXII in Human Thrombi Formed on Atherosclerotic Plaque Material

In whole-blood perfusion experiments over collagen ex vivo, the thrombogenic role of FXII has been explained by activated FXII binding to collagen.[9] This prompted us to determine the

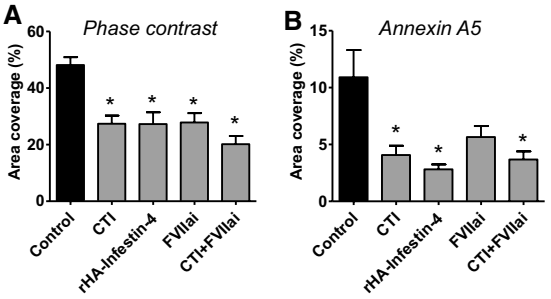

Figure 4. Pharmacological inhibition of factor XII (FXII) suppresses thrombus formation on human plaque material. Blood was perfused over human plaque material, as in Figure 3. Inhibitors corn trypsin inhibitor (CTI; 50 μg/mL) or recombinant infestin-4 human albumin fusion protein (rHA-infestin-4; 250 μg/mL) were present in the collecting tube during blood collection. Incubation with active-site inactivated FVIIa (FVIIai; 10 μg/mL) was for 15 minutes before the experiment. Shown is deposition of all platelets (**A**) and phosphatidylserine-exposing platelets (**B**) at the plaque surface. Data are means±SE (n=5–10); **P*<0.05.

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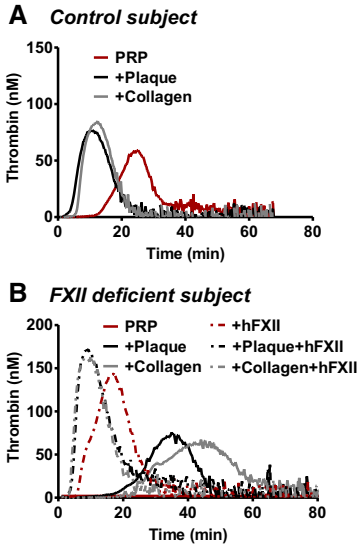

Figure 5. Factor XII (FXII) pathway contributes to plaque-induced thrombin generation. Thrombin generation was measured in platelet-rich plasma (PRP; 1×10^8 platelets/mL), incubated with plaque material (0.28 mg wet tissue weight/mL) or collagen (5 μg/mL), and triggered with $CaCl_2$ (16.6 mmol/L). Preincubation of PRP was with human FXII (hFXII; 375 nmol/L), as indicated. Shown are representative thrombograms with PRP from a control subject (**A**) and an FXII-deficient subject (FXII <1%; **B**). Note the greatly delayed thrombin generation at FXII deficiency.

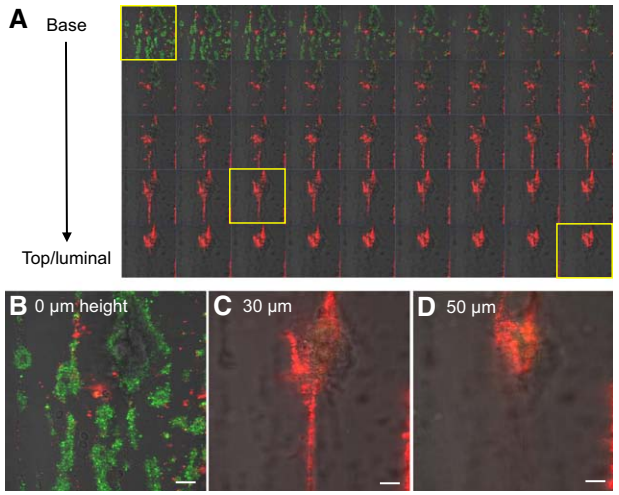

Figure 6. Localization of factor XIIa (FXIIa) on luminal-exposed thrombus parts and on fibrin fibers. Citrate-anticoagulated blood was recalcified with $CaCl_2$/$MgCl_2$ and perfused over plaque material, as indicated for Figure 3. Thrombi on plaques were rinsed with HEPES buffer, blocked with anti-goat serum, and stained with mouse monoclonal antibody against FXIIa, followed by AF647-labeled goat anti-mouse IgG plus AF588–annexin A5, to detect procoagulant phosphatidylserine-exposing platelets. Shown are a gallery of high-resolution overlays of differential interference contrast and confocal images of representative thrombi formed on plaque material at increasing *z* direction (base→top/luminal; **A**) and enlargements at 0 (**B**), 30 (**C**), and 50 (**D**) μm height. Note the increased red FXIIa staining in the *z* direction and the lack of colocalization of FXIIa with green-labeled procoagulant platelets (bars, 20 μm). Data are representative for 3 independent experiments.

localization of FXII in thrombi formed on plaque material. Nonspecific binding sites on the thrombi were blocked by preincubation with anti-goat serum, after which FXIIa was stained with a monoclonal mouse antibody, followed by an AF647-labeled goat anti-mouse IgG. Stainings with an isotype control antibody followed by AF647 goat anti-mouse IgG or AF647 goat anti-mouse IgG alone yielded no fluorescence. Labeling with the anti-FXIIa antibody showed a staining of fibrin fibers (Figure 6). This is compatible with a recent finding that FXIIa can interact with fibrin to modulate clot structure.[21] Stacks of confocal fluorescence images indicated that, at the base of the thrombus near the plaque surface, the staining for FXIIa was punctuated at the outer edges of platelet thrombi and on fibrin fibers (Figure 6). With increasing *z* direction, toward the luminal part of the thrombus, the FXIIa staining became more intense and more evenly distributed. Interestingly, the FXIIa staining did not colocalize with AF568–annexin A5 staining indicative for phosphatidylserine-exposing platelets (Pearson, r=0.02), implying that these procoagulant platelets did not bind FXIIa.

Discussion

In the present article, we revealed a major role of the FXII-driven intrinsic pathway of coagulation in arterial thrombus formation on atherosclerotic plaques. Evidence for the involvement of FXII came from an in vivo model of acute plaque rupture in atherosclerotic mouse arteries using 2 structurally different pharmacological FXIIa inhibitors, from ex vivo flow experiments using blood of *F12*$^{-/-}$ mice and blood of a subject with severe deficiency in FXII (FXII level <1%). The FXII-dependent procoagulant effect of plaque material was also observed as an increase in thrombin generation in the presence of platelets. Interestingly, immunologic staining for FXIIa of thrombi containing platelets and fibrin pointed to an accumulation of FXIIa in the luminal-exposed regions of the thrombus. This likely is relevant, because these top regions are subjected to high shear gradients and hence most prone to embolization.[22] The present findings significantly extend earlier data, using purified or plasma systems, demonstrating that FXIIa can interact with fibrin to modulate clot structure.[21] Taken together, this suggests that local, FXIIa-initiated thrombin and fibrin generation provides stabilization to the regions of a thrombus distant from the vascular TF. Recent studies in which anti-FXII and anti-FXIIa antibodies were found to reduce fibrin formation in an ex vivo perfusion system with human blood provide additional support for this idea.[13,23]

Although the binding sites of FXIIa on platelets have not been elucidated, our confocal microscopic studies indicate that FXIIa does not bind to phosphatidylserine-exposing platelets. This is of interest, because the majority of coagulation factors (FII, FV, FVII, FVIII, FIX, and FX) have been shown to bind to this population of activated platelets.[2] In addition, we and others have shown that both collagen and polyphosphates are able to activate FXII,[5,9] although it needs to be noted that the chain length of platelet-derived polyphosphates is relatively short for optimal FXII activation.[24] Together with the present findings, this would advocate for a combined role of collagen, platelets, and fibrin in activation of the FXII pathway.

Our data indicate that the formation of platelet–fibrin thrombi on immobilized plaque material is similarly affected in blood from *F12*$^{-/-}$, *F11*$^{-/-}$, or *F11*$^{-/-}$/*F12*$^{-/-}$ mice, thus suggesting that the role of FXII in promoting atherothrombosis is

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mediated via activation of FXI. This is in line with several in vivo studies pointing to corresponding roles of FXII and FXI in thrombus formation in artificially damaged nonatherosclerotic mouse vessels.[3,5,10] Consistent with an FXI-dependent role of FXII in arterial thrombosis, an anti-FXI monoclonal antibody that specifically targets FXIIa-mediated FXI activation has been shown to suppress thrombus formation in mice and baboons.[4,25]

Studies by our group and others have elucidated a coagulation-initiating role of atherosclerotic plaques via localized activity of TF, whereas the contact activation pathway was not investigated here.[6,7,26–28] In the present study, inhibitor experiments using FVIIai similarly pointed to a role of TF in the initiation of thrombus formation after plaque rupture in vivo, whereas FXII may be more important at later stages of this process. It is tentative to relate this early effect of the TF–FVIIa pathway and later contribution of the FXII pathway with the fact that FVII deficiency in man is accompanied by severe bleeding, whereas deficiency in FXII is not.[3,14] Notably, our findings contrast to a recently published article showing a lack of effect of CTI on plaque-induced platelet aggregation and thrombin generation in vitro.[8] Differences in methodology and procedures could explain this discrepancy. Of note, in our experiments, FXIIa inhibitors were always added directly during blood taking, and a CTI batch with high enzymatic activity (1.5 U/mL) was used. This procedure was followed to prevent residual ongoing FXIIa activity in the anticoagulated blood samples.

Whereas FXII in man or mouse is not required for normal hemostasis,[3,14] we here demonstrate a promoting role for FXII in pathological thrombus formation after plaque rupture. This is in accordance with clinical evidence that elevated levels of FXII(a) positively associate with arterial thrombosis, ischemic stroke, and coronary heart disease.[29–31] On the contrary, conflicting evidence exists on whether individuals with partly reduced FXII levels have an increased risk of cardiovascular disease (reviewed in Renné et al[15] and Woodruff et al[16]). In this regard, the thrombus-destabilizing effect observed under conditions of reduced FXII activity needs more attention. Patients with a stroke or myocardial infarction mostly have thrombosis of the carotid or coronary artery and may present with symptomatic emboli that potentially give rise to (semi) occlusive thrombi downstream in the vasculature. However, many patients also develop clinically asymptomatic embolization,[32] which remain for the major part without pathological consequences.[33] The latter is in line with the emerging notion that limited instability may be considered as a natural phenomenon in arterial thrombus formation.[22]

Also, in patients with FVII deficiency, thrombotic episodes, particularly deep venous thrombosis, have been reported with an incidence of 3% to 4%.[34] Although the underlying mechanism is unclear, it seems that these are mostly related to surgery and the use of replacement therapy with FVIIa or prothrombin complex concentrate.[34]

In summary, the present results indicate that the coagulation pathways triggered by TF–FVIIa and FXIIa have distinct but complementary roles in atherothrombus formation. The TF–FVIIa pathway contributes to initial thrombus buildup, whereas FXIIa bound to thrombi ensures stability of the thrombus in later phases.

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Disclosures

F. May is an employee of CSL Behring GmbH, Marburg, Germany. The other authors report no conflicts.

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Significance

Atherothrombosis, characterized by atherosclerotic lesion disruption with superimposed thrombus formation, is the major cause of myocardial infarction and ischemic stroke. In this article, we demonstrate that tissue factor– and factor XII (FXII)–triggered coagulation pathways play distinct but complementary roles in coagulation and atherothrombosis. Evidence for this comes from a recently in-house established in vivo model of acute plaque rupture using 2 structurally different pharmacological FXIIa inhibitors with good clinical potential and a compound which blocks tissue factor–FVIIa interactions, from ex vivo flow experiments using blood from *F12*$^{-/-}$ mice and from a subject with a deficiency in FXII (<1%). Furthermore, with an FXIIa antibody, we demonstrate that FXIIa binds to thrombi and fibrin particularly at those areas which are most prone to embolization. We conclude that tissue factor–FVIIa interaction is needed for initial thrombus formation, whereas FXII binds to thrombi, stimulates thrombus formation, and ensures thrombus stability.

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